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Tenascin-C is required for normal Wnt/β-catenin signaling in the whisker follicle stem cell niche

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ABSTRACT

Whisker follicles have multiple stem cell niches, including epidermal stem cells in the bulge as well as neural crest-derived stem cells and mast cell progenitors in the trabecular region. The neural crest-derived stem cells are a pool of melanocyte precursors. Previously, we found that the extracellular matrix glycoproteins tenascin-C and tenascin-W are expressed near CD34-positive cells in the trabecular stem cell niche of mouse whisker follicles. Here, we analyzed whiskers from tenascin-C knockout mice and found intrafollicular adipocytes and supernumerary mast cells. As Wnt/β-catenin signaling promotes melanogenesis and suppresses the differentiation of adipocytes and mast cells, we analyzed β-catenin subcellular localization in the trabecular niche. We found cytoplasmic and nuclear β-catenin in wild-type mice reflecting active Wnt/β-catenin signaling, whereas β-catenin in tenascin-C knockout mice was mostly cell membrane-associated and thus transcriptionally inactive. Furthermore, cells expressing the Wnt/β-catenin target gene cyclin D1 were enriched in the CD34-positive niches of wild-type compared to tenascin-C knockout mice. We then tested the effects of tenascins on this signaling pathway. We found that tenascin-C and tenascin-W can be co-precipitated with Wnt3a. *In vitro*, substrate bound tenascins promoted β-catenin-mediated transcription in the presence of Wnt3a, presumably due to the sequestration and concentration of Wnt3a near the cell surface. We conclude that the presence of tenascin-C in whiskers assures active Wnt/β-catenin signaling in the niche thereby maintaining the stem cell pool and suppressing aberrant differentiation, while in the knockout mice with reduced Wnt/β-catenin signaling, stem cells from the trabecular niche can differentiate into ectopic adipocytes and mast cells.

1. Introduction

Tenascin-C is an extracellular matrix glycoprotein that is found in many stem cell niches, including the subventricular zone of the central nervous system, hematopoietic stem cell niches in bone marrow, corneal limbus and dental pulp (for review, see Chiquet-Ehrismann et al., 2014). Studies of tenascin-C knockout mice reveal possible roles for the tenascin-C of stem cell niches in regulating proliferation, migration and differentiation. For example, in tenascin-C knockout mice, there is a reduction in proliferation within the subventricular zones of the lateral ventricles (Garcion et al., 2001), and in the ependymal layer of the spinal cord, tenascin-C is required for the normal differentiation of astrocytes and their normal migration to white matter (Karus et al., 2011).

Unlike hair follicles, whisker follicles are relatively large, specialized sensory organs that are physically separated from the surrounding dermis by a fibrous capsule (Kim et al., 2011). At least two anatomically distinct populations of stem cells are found within the capsule: keratin-15-positive epidermal cells in the bulge region that are responsible for generating keratinocytes in response to wound healing, and CD34- and nestin-positive stem cells found in the trabecular region between the bulge and the capsule. At least some of the cells from the trabecular region are pluripotent cranial neural crest-derived stem cells that have the potential to differentiate into melanocytes, neurons, glia, smooth muscle and adipocytes in vitro (Amoh et al., 2005; Wong et al., 2006). This region also contains a population of mast cell precursors (Kumamoto et al., 2003). The nestin-positive stem cells can migrate to the dermal papilla along the glassy membrane that surrounds the whisker shaft (Amoh et al., 2012) and contribute both to whisker growth and to the melanocytes that give the whisker color.

We previously found tenascin-C and tenascin-W to be enriched in the trabecular stem cell niche of mouse whisker follicles (Tucker et al.,

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2013). Trabecular niche-derived stem cells cultured in the presence of either tenascin-C or tenascin-W acquire a motile phenotype in vitro, leading us to suggest a role for these glycoproteins in the migration of these stem cells to the dermal papilla. In addition, tenascin-C, but not tenascin-W, promotes to proliferation of these stem cells in vitro (Tucker et al., 2013).

The present study was undertaken to investigate the possible role of tenascins on stem cell differentiation in the whisker follicle, and to provide a potential mechanism for tenascins in regulating differentiation within this and possibly other stem cell niches.

2. Results and discussion

2.1. The absence of tenascin-C leads to the appearance of adipocytes and mast cells inside the whisker follicle

We prepared histological sections of whisker follicles from age and strain matched wild-type and tenascin-C knockout mice. While the morphology of whisker follicles from adult tenascin-C knockout mice is outwardly normal, OsO_4-stained adipocytes were observed in all 5 follicles from 3 tenascin-C knockout mice analyzed, but not in any of the 4 wild-type whisker follicles (Figs. 1 and 2). In addition, there were significantly more mast cells in the loose mesenchyme underlying the cavernous sinus of the tenascin-C knockout mice than in the controls (Figs. 1F and 2).

Whisker follicles are known to serve as a local reservoir of mast cell precursors as it was shown by others that the middle part of dissected whisker follicles is able to produce mast cells in culture when stimulated with the appropriate growth and differentiation factors (Kumamoto et al., 2003). In addition, whisker follicle mesenchymal stem cells, like the cranial neural crest cells from which they are derived, can differentiate into adipocytes *in vitro* (Wong et al., 2006; Billon et al., 2008). Interestingly, both adipogenesis (Ross et al., 2000) and mast cell differentiation (Ichii et al., 2012) are inhibited by canonical Wnt/β-catenin signaling, and Wnt3a inhibits adipocyte (Kanazawa et al., 2005) and mast cell differentiation (Ichii et al., 2012).

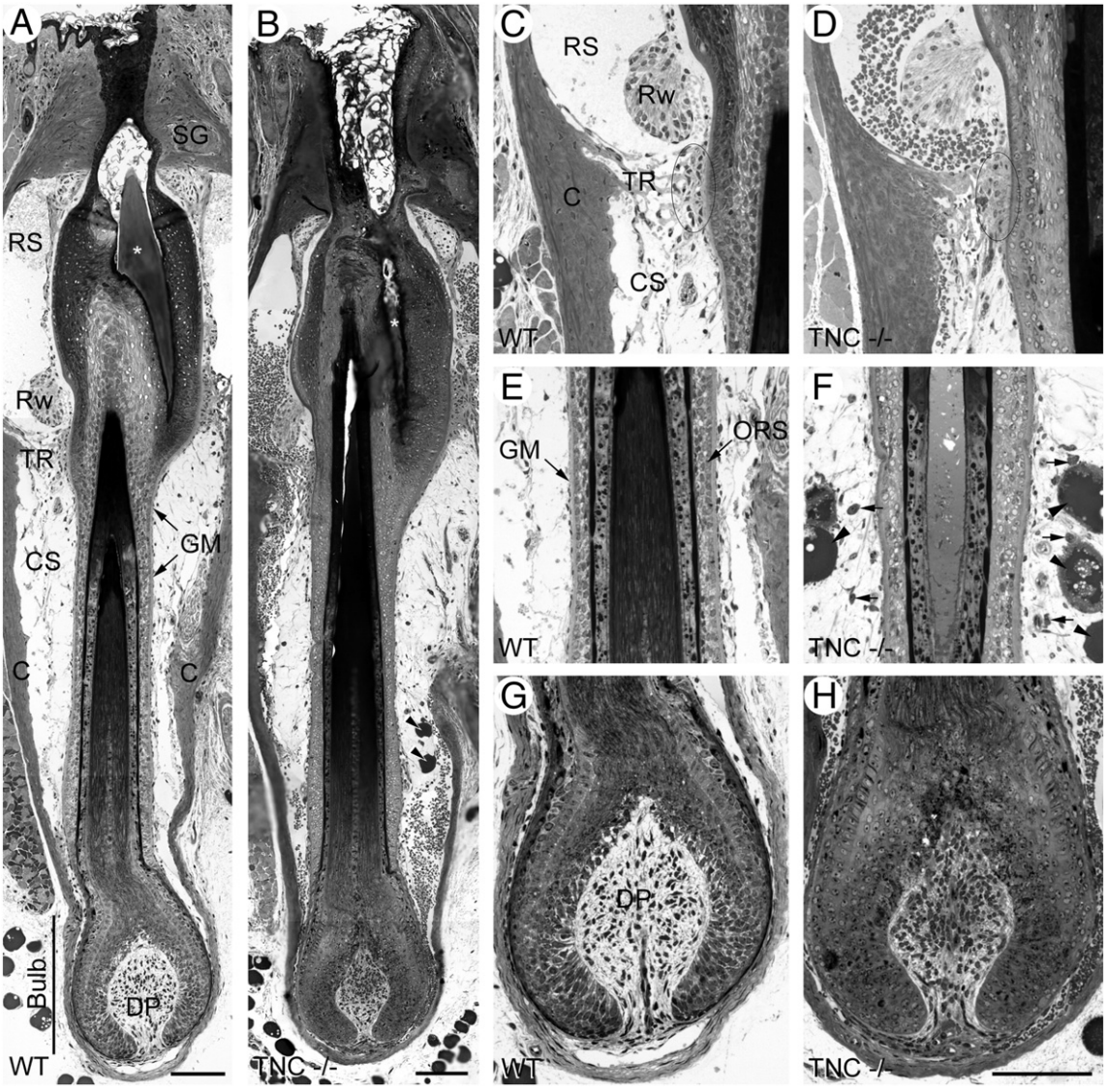


Fig. 1. Comparison between whisker follicles of wild-type and tenascin-C knockout mice. (A) A wild-type whisker follicle stained with toluidine blue and OsO_4. The presence of a non-growing club whisker (asterisk) demonstrates that the whisker is in anagen. (B) A similarly prepared and oriented whisker follicle from a tenascin-C knockout mouse. Darkly stained adipocytes are seen near the cavernous sinus (arrowheads). (C, D) The trabecular region (TR) of a wild-type whisker follicle (C) and a whisker follicle from a tenascin-C knockout mouse (D). Cells found in the region stained with anti-CD34 are indicated with the oval in both sections. (E, F) The whisker shaft from the region midway between the bulb and the trabecular region. The glassy membrane (GM) and outer root sheath (ORS) are indicated. Darkly stained adipocytes (F, arrowheads) are found in the tenascin-C knockout mouse whisker follicles, as are numerous mast cells (arrows). (G, H) The bulbs of the wild-type (G) and tenascin-C knockout mouse (H) are grossly similar. C, fibrous capsule; CS, cavernous sinus; DP, dermal papilla; RS, ring sinus; Rw, Ringwulst; SG, sebaceous gland.

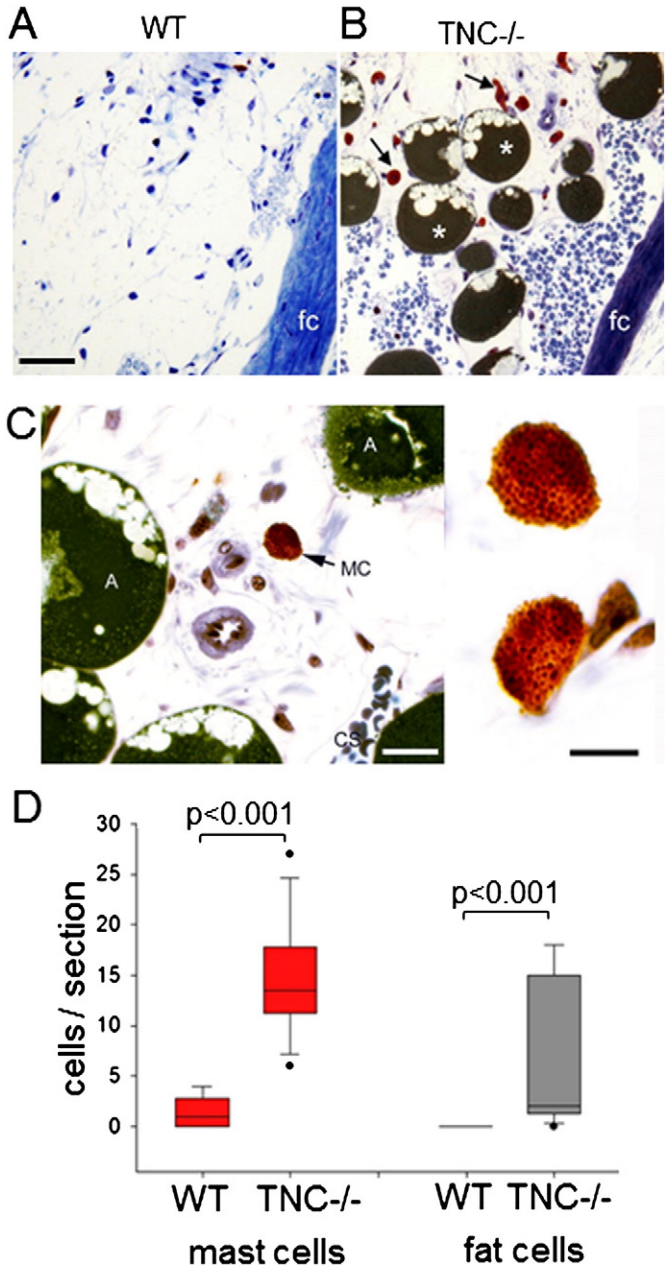


Fig. 2. Ectopic fat and mast cells in whiskers of tenascin-C knockout mice. In contrast to the wild-type whiskers (A), darkly stained adipocytes (B, white asterisks) are found in the tenascin-C knockout mouse whisker follicles, as are numerous toluidine blue-stained mast cells (B, arrows); fc, fibrous capsule; bar, 100 μm. (C) Enlargements of mast cells (MC; arrow) in tenascin-C knockout animals reveal the typical metachromatically stained granules in the further enlargements shown to the right; A, adipocytes; CS, cavernous sinus; bars 20 μm (left panel), 10 μm (right panel). Number of mast cells and adipocytes per section (C) counted in wild-type (WT) or tenascin-C-deficient whiskers (TNC−/−). The difference between WT and TNC−/− mice is highly significant both for the mast and the fat cells as determined by the Mann–Whitney *U* test ($p < 0.001$).

2.2. Wnt/β-catenin signaling is reduced in the trabecular region of TNC −/− whiskers

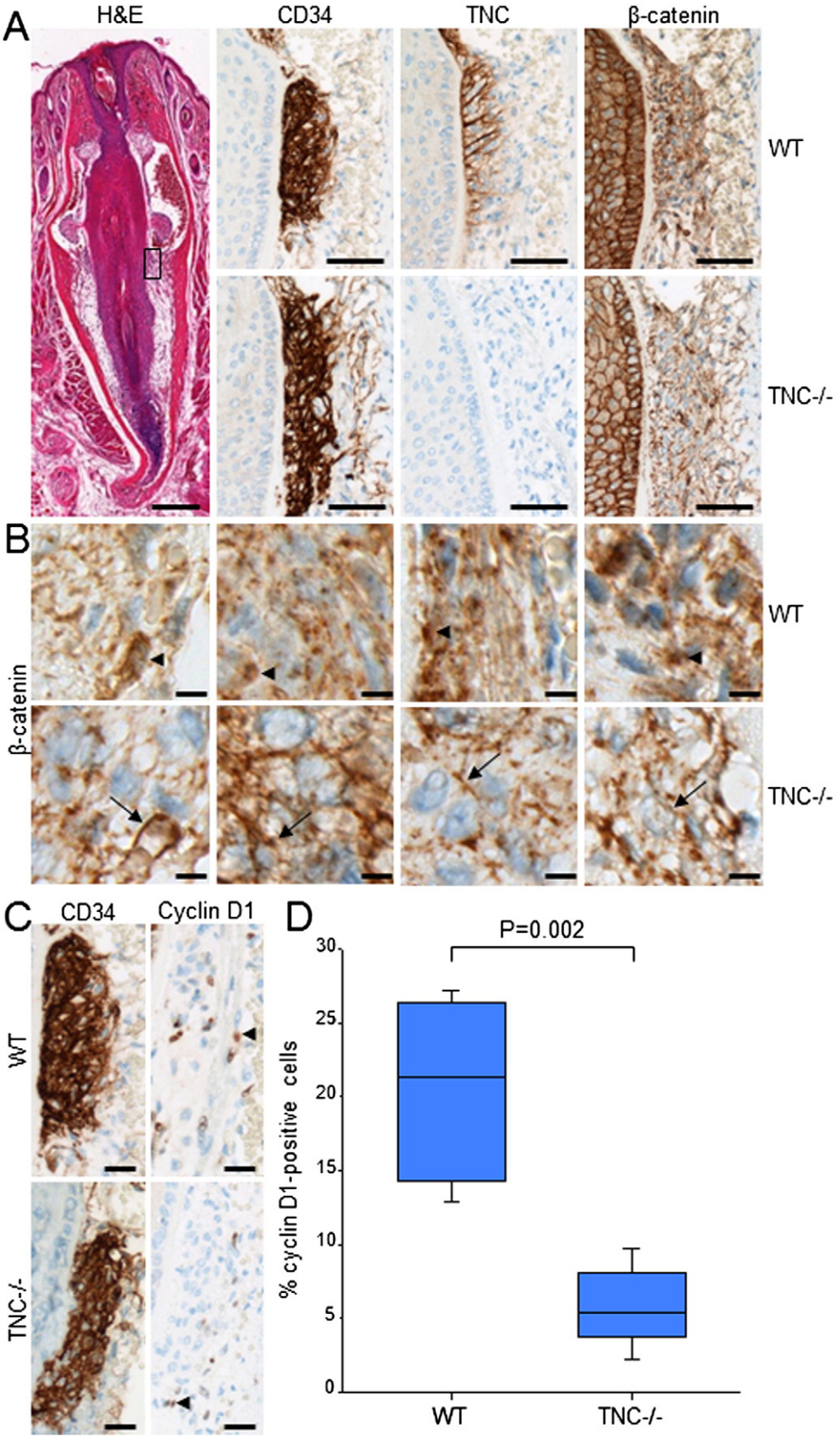
Wnt/β-catenin signaling controls cell fate through regulation of major cell processes such as cell proliferation, death, migration, differentiation and metabolism (MacDonald et al., 2009). In the absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated, followed by degradation by the proteasome. Inversely, when Wnt ligands bind to their frizzled receptors and LRP5/6 co-receptors, β-catenin accumulates in the cytoplasm and nuclei where it activates the expression of target genes (Clevers and Nusse, 2012). In addition, β-catenin is also a component of adherens junctions where it links cadherins to the cytoskeleton to regulate cell adhesion (Jamieson et al., 2012). Therefore, cytoplasmic/nuclear β-catenin is associated with active Wnt signaling, whereas cell membrane-associated β-catenin is associated with low Wnt signaling. In order to test whether indeed Wnt/β-catenin signaling is affected in tenascin-C-deficient mice, we stained whisker follicles for CD34 and β-catenin immunoreactivity in the tenascin-C knockout and wild-type mice. We compared 3 pairs of siblings from heterozygous crossings and found that the subcellular distribution of β-catenin differed between wild-type and knockout mice (Fig. 3). In serial sections of whiskers, we stained for CD34 to identify the stem cells in the trabecular region. Adjacent sections were immunostained for tenascin-C and for β-catenin. The anti-β-catenin staining in wild-type mice was diffuse throughout the cytoplasm as well as in some nuclei. These results are consistent with the β-catenin immunostaining presented by others in whiskers of 5-day and 1-month-old wild-type C57B6 mouse whisker follicles (Ridanpaa et al., 2001). Although not addressed in that paper, clear cytoplasmic β-catenin immunostaining can be observed in the trabecular regions of the whisker sections displayed (Ridanpaa et al., 2001). In contrast, the β-catenin staining pattern was clearly different in the tenascin-C knockout mice where it was well defined along cell membranes in the CD34-positive stem cell niche (Fig. 3A right panels). Enlargements of the β-catenin staining in this niche of 4 additional whiskers from 3 different mice of each genotype are shown in Fig. 3B. This confirmed that in the CD34-positive cells of wild-type animals, Wnt/β-catenin signaling is active (cytoplasmic and nuclear immunostaining), while it is attenuated in the tenascin-C-deficient mice (membrane-associated immunostaining). To corroborate these findings, we also investigated the expression of the Wnt/β-catenin target gene cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999). Indeed, there was a 4-fold higher number of cyclin D1-positive cells in the CD34-positive niche area of wild-type versus tenascin-C knockout mice, supporting our finding of a positive impact of the presence of tenascin-C on Wnt/β-catenin signaling. Since cyclin D1 is known to be involved in G1/S phase transition of the cell cycle (Stacey, 2003), this result fits well with our previous observation that tenascin-C increases the proliferation of cultured whisker stem cells (Tucker et al. 2013).

2.3. Tenascin-C and tenascin-W bind Wnt3a and modulate Wnt3a-mediated gene regulation by β-catenin

We therefore considered that in the normal situation, tenascin-C may positively influence Wnt signaling and that absence of tenascin-C may lead to reduced Wnt signaling, thereby causing ectopic adipocyte and mast cell differentiation from whisker follicle stem cells. Thus, we tested whether tenascin-C interacts with Wnt3a, a prototypic Wnt ligand for canonical Wnt/β-catenin signaling (Willert and Nusse,

Fig. 3. Tenascin-C expression is associated with active Wnt/β-catenin signaling in the CD34-positive whisker stem cell niche. (A) Hematoxylin and eosin-stained section of a mouse whisker follicle (left panel) showing the CD34-positive stem cell niche (rectangle). Immunoperoxidase detection (brown) of CD34, tenascin-C (TNC) and β-catenin in the CD34-positive stem cell niches of wild-type (WT) and TNC−/− mice (right panels). Scale bar for H&E panel = 200 μm, scale bar for immunostainings = 50 μm. (B) Enlargements of β-catenin stainings within CD34-positive stem cell niches of WT and TNC−/− mice. Three WT and 3 TNC−/− mice are showed, and each panel represents an independent whisker follicle. Scale bar = 5 μm. (C) Representative images of cyclin D1 immunostaining (arrowheads) in CD34-positive stem cell niches of wild-type and TNC−/− mice. Scale bar = 20 μm. (D) Quantification of cyclin D1-positive cells in CD34-positive stem cell niches of matched pairs of adult siblings of 3 wild-type and 3 TNC−/− mice. Percentage of cyclin D1-positive cells in CD34-positive stem cell niches is significantly reduced in TNC−/− compared to wild-type mice, as determined by a Mann–Whitney *U* test ($p = 0.002$).

2012). Since tenascin-W is also present in the CD34-positive whisker niche (Tucker et al. 2013), we included tenascin-W in our experiments as well. Wnt3a conditioned medium was incubated with either purified recombinant tenascin-C or tenascin-W. Both tenascins are present in anti-Wnt3a precipitates following Wnt3a immunoprecipitation, but not in precipitates using control IgG (Fig. 4A). For an additional control, purified fibronectin did not co-immunoprecipitate with Wnt3a (Fig. 4A).



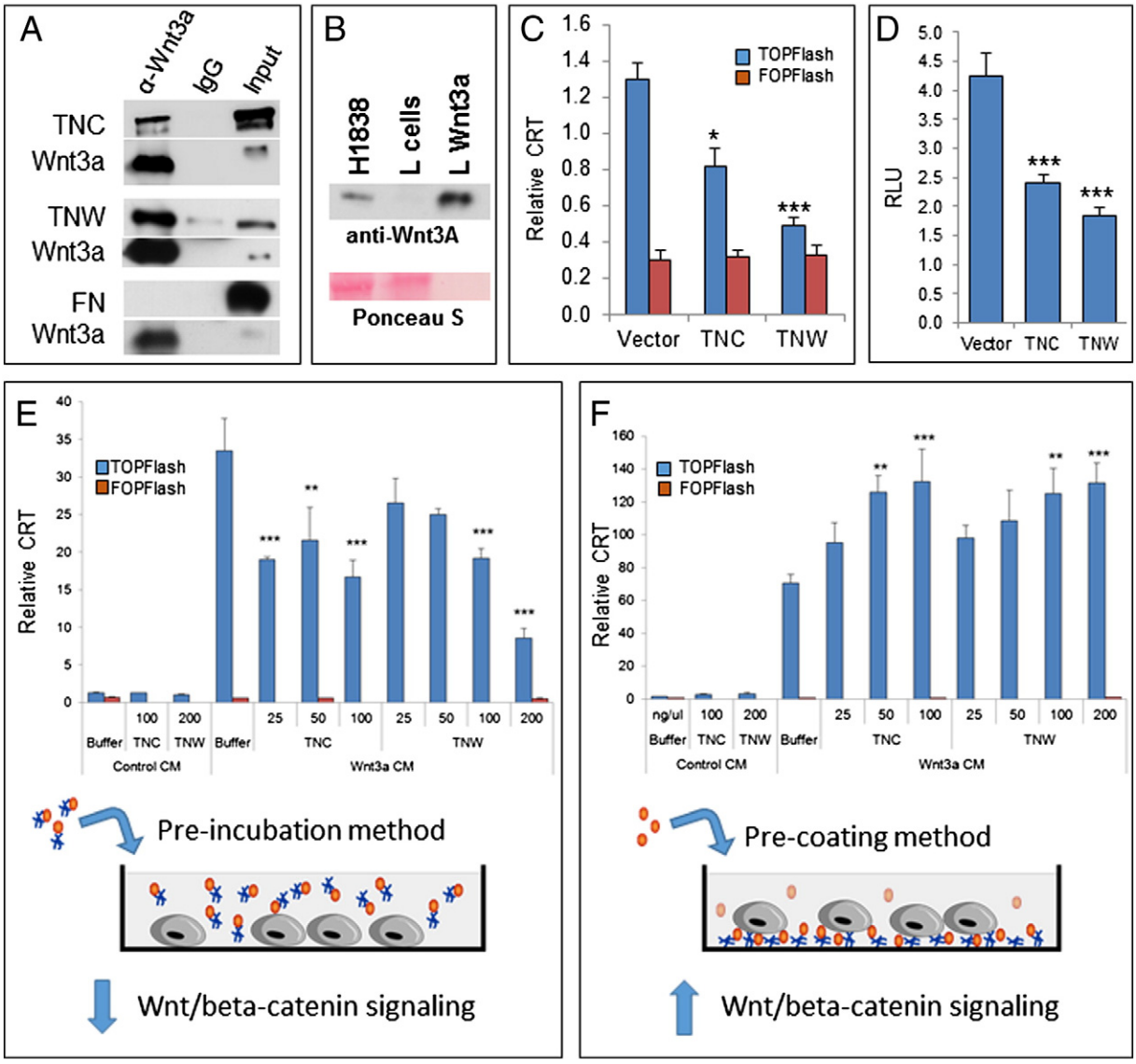


Fig. 4. Tenascin-C and tenascin-W bind Wnt3a and modulate Wnt/β-catenin signaling. (A) Recombinant tenascin-C (TNC) and tenascin-W (TNW) bind soluble Wnt3a. Conditioned medium from L Wnt3a cells was incubated with either recombinant TNC, TNW or fibronectin (FN; negative control) overnight at 4 °C, and 25 μl of each mixture was loaded as input. Wnt3a was immunoprecipitated with anti-Wnt3a and the precipitates were loaded on gels, transferred to membranes where TNC, TNW or FN were detected by anti-TNC, anti-TNW or anti-FN, respectively. The lower part of the membrane was used to detect Wnt3a with anti-Wnt3a as control. While TNC and TNW were present in the anti-Wnt3A precipitates, this was neither the case with FN, nor in the precipitates using control IgG. (B) H1838 cells express endogenous Wnt3a. Total cell lysates from H1838 (50 μg), L (50 μg) and L Wnt3a cells (5 μg) were subjected to electrophoresis and immunobloted with anti-Wnt3a. Ponceau staining was used as loading control. (C) TNC and TNW overexpression in H1838 cells inhibits their basal Wnt/β-catenin signaling activity. β-Catenin-T-cell factor regulated Transcription (CRT) assays using a β-catenin-TCF reporter driven by wild-type (Super 8x TOPFlash) or a negative control with mutated TCF binding sites (Super 8x FOPFlash) is shown. (D) TNC and TNW overexpression in H1838 cells inhibits cyclin D1 promoter activity. Reporter gene assays using a cyclin D1 promoter driving luciferase expression is shown. RLU, relative light unit. (E–F) Recombinant TNC and TNW modulate Wnt3a-induced Wnt/β-catenin signaling activity in H1838 cells. Pre-incubation method (E): control conditioned medium (CM) or Wnt3a CM were incubated for 24 h with buffer or increasing concentrations of recombinant TNC or TNW, before being used to treat cells transfected with the CRT reporters for 16 h. A scheme of the experimental setup is given below the bar graph. Pre-coating method (F): cells were seeded on wells pre-coated with increasing concentrations of recombinant TNC or TNW. Then cells were transfected with the CRT reporter before treating them with either control CM or Wnt3a CM for 16 h. A scheme for the experimental setup is given below the bar graph. All reporter assays (C–F) are representative of three independent experiments performed in triplicate and normalized to Renilla luciferase activity (mean ± SD). Asterisks denote a significant difference compared with vector (C–D) or buffer/Wnt3a CM condition (E–F), as determined by one-way ANOVA with *post hoc* Tukey–Kramer test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Next, we tested whether the presence of tenascin-C and/or tenascin-W has an effect on canonical Wnt signaling through β-catenin-induced gene transcription using a luciferase reporter under control of 8 TCF/LEF binding sites (TOPFlash). As a control, the same reporter with mutated binding sites was used (FOPFlash). The H1838 cell line was used for this study since endogenous Wnt3a could be detected in H1838 cell lysate (Fig. 4B) and after transfection, the TOPFlash plasmid, but not the control FOPFlash construct, was transactivated. When we co-transfected the cells with these reporters together with either empty pCEP-Pu or pCEP-Pu encoding tenascin-C or tenascin-W, we found that overexpression of tenascins in H1838 cells attenuated the β-catenin-induced transcription of the TOPFlash plasmid, while there was no effect on the basal activity of the control plasmid (Fig. 4C).

Since cyclin D1 is known to be a target gene of Wnt/β-catenin signaling (Shtutman et al., 1999; Tetsu and McCormick, 1999), we investigated the effect of overexpression of tenascin-C and tenascin-W on cyclin D1 promoter activity using a luciferase reporter under control of cyclin D1 promoter. The same inhibitory effect of both tenascin-C and tenascin-W overexpression was observed on cyclin D1 promoter activity (Fig. 4D). Thus, using this experimental design, secretion of tenascin-C or tenascin-W by the H1838 cells appears to inhibit Wnt signaling.

In order to investigate whether the binding of the tenascins to Wnt3a would influence the reporter activities, we analyzed the effect of recombinant tenascins and Wnt3a on β-catenin-mediated transcription. We either incubated tenascins in solution with Wnt3a before adding this mixture to the cells (pre-incubation method) or coated tenascins on tissue culture plates before plating the cells transfected with the reporter constructs in Wnt3a containing medium (pre-coating method). In cells cultured in the presence of soluble tenascins incubated with Wnt3a conditioned medium, the reporter activity was attenuated by the addition of increasing amounts of tenascins (Fig. 4E). The opposite was observed when cells were plated on tenascin-coated substrata: bound tenascin led to a concentration dependent increase in reporter activity in the presence of Wnt3a (Fig. 4F). From these results, we conclude that soluble tenascins sequester Wnt3a in the medium away from the cells, while binding of Wnt3a to immobilized tenascins leads to a high local concentration of Wnt3a in proximity to the cell surface, which increases Wnt/β-catenin signaling. Models depicting the proposed actions of tenascins in mediating Wnt3a signaling are shown below the respective graphs in Fig. 4D and E. These results are reminiscent of our earlier findings that Wnt signaling is upregulated in glioblastoma cells cultured on substrata containing tenascin-C (Ruiz et al., 2004).

We propose the following mode of action of tenascins in the stem cell niche of the trabecular region (Fig. 5): the presence of tenascin-C assures active Wnt/β-catenin signaling, thereby maintaining the stem cell pool, while in the knockout mice with reduced Wnt/β-catenin signaling, the stem cells can differentiate into ectopic adipocytes and supernumerary mast cells. As tenascin-W can bind to Wnt3a as well, the roles of these tenascins in the stem cell niche may be, at least in part, redundant. Once tenascin-W knockout mice become available, this can be tested.

Tenascin-C and tenascin-W have been observed in several other stem cell compartments in conjunction with Wnt/β-catenin signaling. For example, tenascin-W is highly expressed in the periosteum, which is the stem cell compartment of osteoblasts (Scherberich et al., 2004). The addition of tenascin-W to osteoblast cultures suppresses Wnt/β-catenin signaling accompanied by an inhibition of cell proliferation (Kimura et al., 2007). Co-localization of tenascin-C and β-catenin activation has also been observed in a specialized stem cell niche that enables repetitive tooth renewal in the alligator (Wu et al., 2013). In the chicken, tenascin-C has been found to be expressed together with Wnt3a in the dermal papilla at the follicle base during the feather cycle (Lin et al., 2013). The transcript profiling of limbal stem cells revealed co-expression of tenascin-C with genes that were linked by Gene ontology and network analysis to Wnt and TGF-β signaling (Nakatsu et al., 2013). In the retinal stem cell compartment, the absence of tenascin-C caused the emergence of early post-mitotic neurons and again Wnt signaling was suggested to play a role (Besser et al., 2012). Finally, in breast cancer, tenascin-C was shown to be part of the metastatic niche enhancing the expression of the Wnt target gene LGR5 (Oskarsson et al., 2011). Therefore, tenascin-C and tenascin-W may be of general importance for the microenvironmental effect on Wnt signaling, not only in stem cell niches during normal development and tissue homeostasis but possibly also in the tumor microenvironment.

3. Materials and methods

3.1. Analysis of tenascin-C knockout mice

Mystacial whisker pads from 3 adult 129/Sv tenascin-C knockout mice and an age and strain-matched control were fixed in 4% paraformaldehyde and post-fixed in 1% OsO_4, which stains unsaturated fats in adipocytes black (Hayes et al., 1963). The specimens were embedded in Poly/Bed resin (Polysciences), and 50–150 1.5 μm serial sections were cut using a Leica EM UC6 ultramicrotome and stained with toluidine blue, which stains mast cells metachromatically (Williams et al., 1959). Mast cells and adipocytes were counted in the loose mesenchyme between the bulb and the ring sinus in 3 sections each from 4

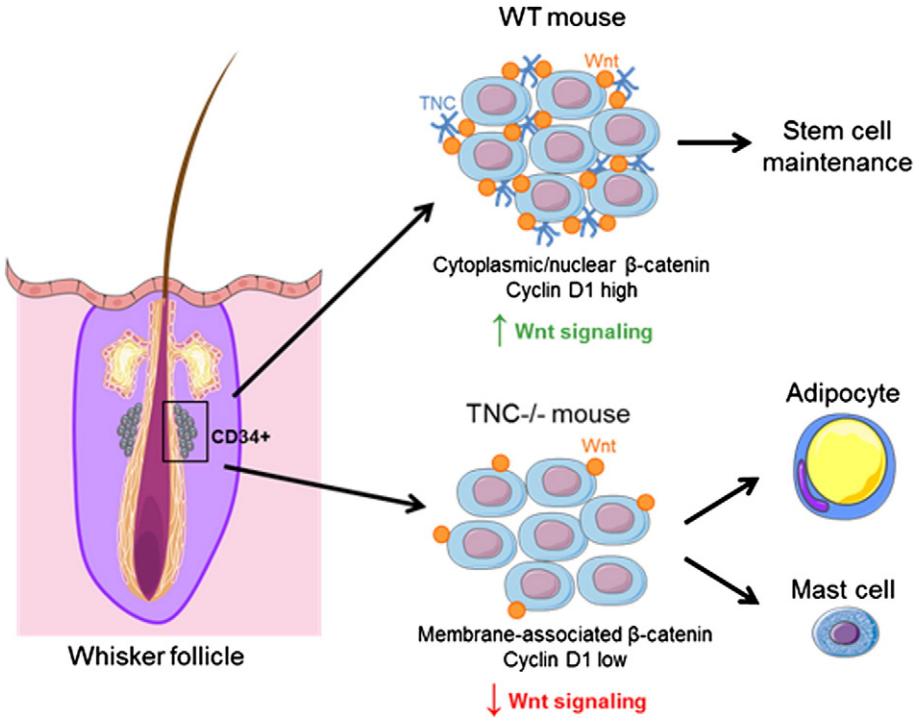


Fig. 5. Model for the role of tenascin-C in mouse whisker follicles. In wild-type (WT) mice, tenascin-C (TNC) is present in the niche of CD34-positive stem cells where it binds Wnt ligands leading to a localized increased concentration of Wnt factors. As a result, Wnt/β-catenin signaling is activated and the stem cell reservoir is maintained. In TNC −/− mice, the absence of TNC in the CD34-positive stem cell niche leads to reduced binging of Wnt ligands and thus to decreased Wnt/β-catenin activity. As a consequence, stem cells mistakenly differentiate into adipocyte and/or mast cell.

follicles from tenascin-C knockout mice and 4 control follicles and compared with a Mann–Whitney *U* test.

For the determination of Wnt/β-catenin signaling in whiskers follicles, mystacial whisker pads from 3 pairs of adult siblings from heterozygous crossings (3 WT mice and 3 adult 129/Sv tenascin-C knockout mice) were fixed for 24 h in Shandon™ Formal-Fixx™ (Thermo Scientific). Then specimens were embedded in MEDITE Pure™ Paraffin (Medite), and 3 μm serial sections were cut using a Microm HM 355 S microtome (Thermo Scientific). After immunostaining for CD34, tenascin-C and β-catenin (see 3.4.) images were acquired using a Nikon Eclipse E800 photomicroscope (4X Plan Fluor, NA 0.13; 20X Plan Fluor, NA 0.50) equipped with an EXi AQUA CCD camera and a MicroPublisher 5.0 digital color camera (Q Imaging) using Q Capture Pro 7 software (Q Imaging). To quantify cyclin D1-positive cells in the whisker follicles, serial sections were stained for CD34 and cyclin D1 (see Section 3.4). All cells within the CD34-positive area were counted and the percentage of cyclin D1-positive cells were manually detected using Fiji (Eliceiri et al., 2012) with the cell counter plugin (ImageJ).

3.2. cDNAs and recombinant proteins

Human tenascin-C and tenascin-W cDNAs were cloned into the eukaryotic expression vector pCEP-Pu and expressed and purified as described previously (Degen et al., 2007). Human fibronectin was obtained from BD Biosciences (#356008). Super 8x TOPFlash (Addgene plasmid 12456) and Super 8x FOPFlash (Addgene plasmid 12457) reporters were from R. Moon (Veeman et al., 2003). The cyclin D1 promoter reporter D1Δ-944pXP2 was from J. Pouysségur (Lavoie et al., 1996). The normalization Renilla luciferase vector pGL4.79[*hRluc*/Neo] was obtained from Promega.

3.3. Cell culture and reporter assays

The human non-small cell lung cancer cell line H1838 was cultured in RPMI plus 10% FBS. The conditioned media from parental L cells and the Wnt3a conditioned medium were collected as recommended by the American Type Culture Collection and the R. Nusse's lab website (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/).

For reporter assays using Super 8x TOP/FOPFlash reporters cells (25000/well) were transfected in 24-well plates with FuGENE 6 (Promega). Per transfection, Super 8x TOP/FOPFlash reporters (15 ng each) or Cyclin D1 promoter reporter (100 ng) were cotransfected with 250 ng of empty pCEP-Pu, tenascin-C or tenascin-W vectors and 1 ng pGL4.79[*hRluc*/Neo] expressing Renilla luciferase as a normalization standard. Where indicated, cells were treated for 16 h with L conditioned medium or Wnt3a conditioned medium ½ diluted in DMEM plus 10% FBS at 24 h post-transfection. Luciferase activity was measured in a microplate luminometer (Mithras LB 940, Berthold Technologies) 40 h post-transfection using the Dual Luciferase Reporter Assay System (Promega). For the pre-coating method, wells were pre-coated 1 h at 37 °C with PBS-Tween 0.01% (control) or increasing concentrations of recombinant TNC or TNW, washed 3 times with PBS, saturated 30 min at 37 °C with PBS-BSA 3% and washed again once with PBS, before seeding cells. For the pre-incubation method, control conditioned medium or Wnt3a conditioned medium were incubated 24 h with PBS-Tween 0.01% (control) or increasing concentrations of recombinant tenascin-C or tenascin-W before adding it to the cell cultures.

3.4. Antibodies and immunological methods

Both monoclonal mouse anti-human tenascin-C (B28-13) and anti-human tenascin-W (56O) antibodies were made in our laboratory (Schenk et al., 1995; Degen et al., 2008). Mouse monoclonal anti-human fibronectin (Ab-3) was obtained from Calbiochem (clone FBN11, #CP70).

For co-immunoprecipitations, recombinant tenascin-C (1.38 nM), tenascin-W (2.78 nM) or fibronectin (2.78 nM) was incubated with pre-cleared Wnt3a conditioned medium and incubated with either rabbit anti-mWnt3a (C64F2, Cell Signaling) or rabbit IgG (sc-2027, Santa Cruz Biotechnology) on a rotary wheel at 4 °C overnight. Then Dynabeads (Invitrogen) protein G (for tenascin-C) or protein A (for tenascin-W) were added to immunocomplexes and incubated at 4 °C for 1 h. After washing with RIPA buffer (Tris–HCl 50 mM, pH 7.4; 1% Triton X-100; 25 mM Hepes; 150 mM NaCl; 0.2% Sodium deoxycholate, 5 mM $MgCl_2$), complexes were eluted in denaturing sample buffer, resolved by 4%–15% SDS–PAGE gradient (Bio-Rad) and immunoblotted. Signal from immunoblots was detected by enhanced chemiluminiscence and exposed to Super RX films (Fujifilm) or acquired with ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Immunohistochemistry experiments were performed on a Ventana Discovery XT instrument (Roche Diagnostics) with the following antibodies: monoclonal rat anti-tenascin (mTn12; (Aufderheide and Ekblom, 1988), monoclonal rat anti-CD34 (Abcam ab8158), polyclonal rabbit anti-β-catenin (Cell Signaling #9587) and monoclonal rabbit anti-cyclin D1 (Abcam ab16663). The procedure Research IHC DAB Map XT was used with CC1 pre-treatment (mild for anti-TNC and anti-cyclin D1, and standard for anti-CD34 and anti-β-catenin). Antibodies (anti-TNC 1:200, anti-CD34 1:1000, anti-β-catenin 1:100 and anti-cyclin D1 1:50) were applied manually and incubated for 1 h at 37 °C. In addition, a blocker (Discovery® Antibody Block, Ventana) was applied with anti-TNC. A linker monoclonal rabbit-anti-rat antibody (Abcam ab125900) was applied for 20 min for anti-TNC and anti-CD34 antibodies. Antibody–antigen complexes were detected with ImmPRESS anti-rabbit Ig (peroxidase) polymer reagent (Vector laboratories MP-7401) applied manually and incubated for 32 min. Sections were finally counterstained with Hematoxylin II and bluing reagent (Roche Diagnostics) for 4 min, dehydrated and mounted with Neo-Mount (Merck UN 1268).

For Fig. 4 images were acquired with an Nikon Eclipse 80*i* microscope connected to a Leica DFC420 camera using a 4X (Plan Apo, NA 0.2) objective for the H&E panel and a 40X (Plan Fluor, NA 0.75) objective for all other panels. Image processing (calibration and zoom) was performed with the IMS software (Imagic).

3.5. Software

Statistical tests were performed using InStat 3 (GraphPad) or SigmaPlot 12.5 (Systat Software Inc.). Illustrations were created using Servier Medical Art Powerpoint Image Bank (Servier, http://www.servier.com/Powerpoint-image-bank).

Contributors

R.P.T., J.S., I.H. and R.C.-E. developed the concepts and approach. R.P.T., J.S., I.H., D.Z. and S.B. performed experiments and analyzed data. R.P.T., I.H. and R.C.-E. prepared the manuscript.

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